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Abstract: Defects of N-linked glycosylation represent diseases with multiple organ involvements that are classified as congenital disorders of glycosylation (CDG). In recent years, several CDG types have been attributed to defects of dolichol-linked oligosaccharide assembly in the endoplasmic reticulum. The profiling of [3H]mannose-labeled lipid-linked oligosaccharides was instrumental in identifying most of these glycosylation disorders. However, this method is poorly suited for the identification of short lipid-linked oligosaccharide biosynthesis defects. To adequately resolve deficiencies affecting the first steps of lipid-linked oligosaccharide formation, we have used a non-radioactive procedure employing the fluorescence detection of 2-aminobenzamide-coupled oligosaccharides after HPLC separation. By applying this method, we have detected the accumulation of dolichylpyrophosphate-GlcNAc2 in a previously untyped CDG patient. The accumulation pattern suggested a deficiency of the ALG1 1,4 mannosyltransferase, which adds the first mannose residue to lipid-linked oligosaccharides. This was supported by the finding that this CDG patient was compound heterozygous for three mutations in the ALG1 gene, leading to the amino acid substitutions S150R and D429E on one allele and S258L on the other. The detrimental effect of these mutations on ALG1 protein function was demonstrated in a complementation assay using *alg1* *Saccharomyces cerevisiae* yeast mutants. The ALG1 mannosyltransferase defect described here represents a novel type of CDG, which should be referred to as CDG-Ik

DOI: <https://doi.org/10.1093/hmg/ddh050>

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ZORA URL: <https://doi.org/10.5167/uzh-153859>

Journal Article

Published Version

Originally published at:

Grubenmann, C E; Frank, C G; Hülsmeier, A J; Schollen, E; Matthijs, G; Mayatepek, E; Berger, E
G; Aebi, M; Hennet, Thierry (2004). Deficiency of the first mannosylation step in the N-glycosylation
pathway causes congenital disorder of glycosylation type Ik. *Human Molecular Genetics*, 13(5):535-542.
DOI: <https://doi.org/10.1093/hmg/ddh050>

Deficiency of the first mannosylation step in the N-glycosylation pathway causes congenital disorder of glycosylation type Ik

Claudia E. Grubenmann^{1,†}, Christian G. Frank^{2,†}, Andreas J. Hülsmeier¹, Els Schollen³, Gert Matthijs³, Ertan Mayatepek⁴, Eric G. Berger¹, Markus Aebi² and Thierry Hennot^{1,*}

¹Institute of Physiology, University of Zürich, Winterthurerstrasse 190, 8057 Zürich, Switzerland, ²Institute of Microbiology, Swiss Federal Institute of Technology, Schmelzbergstrasse 7, 8092 Zürich, Switzerland, ³Center for Human Genetics, Catholic University Leuven, 3000 Leuven, Belgium and ⁴Department of General Pediatrics, University Children's Hospital, University of Düsseldorf, Moorenstrasse 5, 40225 Düsseldorf, Germany

Received October 29, 2003; Revised December 15, 2003; Accepted December 22, 2003

Defects of N-linked glycosylation represent diseases with multiple organ involvements that are classified as congenital disorders of glycosylation (CDG). In recent years, several CDG types have been attributed to defects of dolichol-linked oligosaccharide assembly in the endoplasmic reticulum. The profiling of [³H]mannose-labeled lipid-linked oligosaccharides was instrumental in identifying most of these glycosylation disorders. However, this method is poorly suited for the identification of short lipid-linked oligosaccharide biosynthesis defects. To adequately resolve deficiencies affecting the first steps of lipid-linked oligosaccharide formation, we have used a non-radioactive procedure employing the fluorescence detection of 2-aminobenzamide-coupled oligosaccharides after HPLC separation. By applying this method, we have detected the accumulation of dolichylpyrophosphate-GlcNAc₂ in a previously untyped CDG patient. The accumulation pattern suggested a deficiency of the *ALG1* β 1,4 mannosyltransferase, which adds the first mannose residue to lipid-linked oligosaccharides. This was supported by the finding that this CDG patient was compound heterozygous for three mutations in the *ALG1* gene, leading to the amino acid substitutions S150R and D429E on one allele and S258L on the other. The detrimental effect of these mutations on *ALG1* protein function was demonstrated in a complementation assay using *alg1 Saccharomyces cerevisiae* yeast mutants. The *ALG1* mannosyltransferase defect described here represents a novel type of CDG, which should be referred to as CDG-Ik.

INTRODUCTION

Inborn errors of glycosylation are a rapidly expanding family of genetic diseases encompassing various clinical entities. Deficiencies of N-linked glycosylation, also referred to as congenital disorders of glycosylation (CDG), represent multi-systemic diseases with central and peripheral nervous involvements often associated with endocrine and coagulation disorders (1–3). Defects of O-mannosylation have recently been associated with Walker–Warburg syndrome (4) and different forms of muscular dystrophies like Muscle–Eye–Brain disease (5), Fukuyama congenital muscular dystrophy (6) and MDC1D (7). Dermatological (8) and bone disorders like

hereditary multiple exostoses (9) are caused by alterations of glycosaminoglycan chain formation.

The broad symptomatology of glycosylation disorders and the tremendous structural diversity of glycoconjugates render the identification of glycosylation defects a difficult task. A simple test consisting of the isoelectric focusing separation of serum transferrin (10), a glycoprotein normally bearing two N-glycan chains, has greatly facilitated the detection of N-linked glycosylation defects. While this assay reliably identifies underglycosylation related to lowered sialylation, it lacks the resolution needed to pinpoint specific enzymatic defects. In combination with western blot analysis of serum transferrin, the isoelectric focusing test only allows discrimination between

*To whom correspondence should be addressed at: Institute of Physiology, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland. Tel: +41 16355080; Fax: +41 16356814; Email: thennet@access.unizh.ch

[†]The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

defects of oligosaccharide assembly and transfer, defined as CDG-I (11) and defects of N-linked glycan processing grouped as CDG-II.

Analysis of [^3H]mannose-labeled lipid-linked oligosaccharides (LLO) has enabled the characterization of several types of CDG-I caused by alterations of dolichylpyrophosphate (*DolPP*)-linked $\text{GlcNAc}_2\text{Man}_9\text{Glc}_3$ assembly at the membrane of the endoplasmic reticulum (ER) (12,13). However, a significant group of CDG-I patients, diagnosed by serum transferrin analysis, do not present any accumulation of incomplete oligosaccharides when using the established LLO profiling method. In fact, this method lacks the sensitivity required to detect accumulation of short LLOs such as those lacking mannose or those including only few mannose units. To remedy this problem, we have adapted a method based on the fluorescence detection of total cellular LLOs derivatized with 2-aminobenzamide (2AB). Using this novel approach, we have screened a group of untyped CDG-I patients and identified a case characterized by the accumulation of *DolPP*- GlcNAc_2 . Genetic analysis confirmed in this patient a deficiency of the *ALG1* $\beta 1,4$ mannosyltransferase, which catalyzes the first mannosylation step in the assembly of N-glycans.

RESULTS

The male patient FR was born after an uncomplicated pregnancy and presented early on with minimal signs of psychomotor development. There were no dysmorphic features present. The patient was affected by multiple intractable seizures, generalized muscular hypotonia and blindness. By 6 months of age, sepsis and pneumonia required prolonged hospitalization. Before 1 year of age, liver dysfunction became prominent as well as coagulation problems related to low antithrombin-III (12% of normal values). MRI scan of the brain was normal. The combination of developmental symptoms and coagulation disorder suggested a possible case of CDG. Isoelectric focusing of serum transferrin showed a typical CDG-I pattern with decreased tetrasialo and with increased disialo and asialotransferrin (Fig. 1), thus confirming the diagnosis. Phosphomannomutase activity measured in the patient fibroblasts yielded an intermediate value of 2.2 mU/mg protein (normal range 5.3 ± 1.7 mU/mg protein), which led to the sequencing of the candidate *PMM2* gene. The patient was found to be heterozygous for a point mutation causing the amino acid substitution E197A on the maternal allele. The *PMM2* E197A substitution has been found in combination with two genuine mutations in three other confirmed CDG-Ia patients. Hence, it is probably a polymorphism (unpublished data). It was concluded that it is unrelated to the disease in this patient.

To determine whether a defect of oligosaccharide assembly was the source of the glycosylation disorder, we analyzed the LLO profile in the patient fibroblasts after [^3H]mannose labeling using a procedure established previously (14–16). This analysis failed to reveal any accumulation of incomplete LLO structure (Fig. 2A–C). The level of [^3H]mannose incorporation was very low, indicating a limited LLO pool in the patient's cells. The detection of newly formed N-linked oligosaccharides (NLO) after 60 min labeling with [^3H]mannose showed the same profile as that found in cells from healthy controls (Fig. 2D–F), with peaks corresponding to

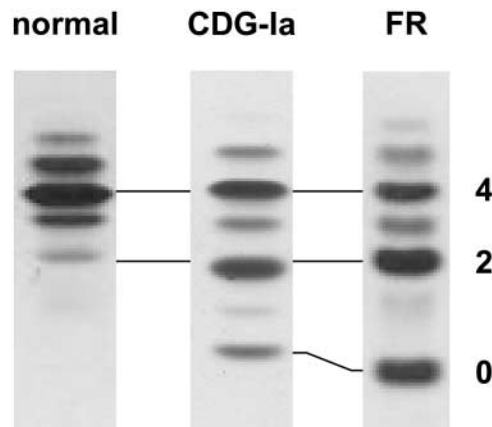


Figure 1. Isoelectric focusing of serum transferrin. Samples from a healthy control serum (normal), from a CDG-Ia control and from patient FR are shown. The number of negative charges is given at the right. The two glycan chains of normal transferrin contain mainly four sialic acids, whereas bands corresponding to disialo- and asialo-transferrin are visible in the FR sample.

$\text{GlcNAc}_2\text{Man}_8$, $\text{GlcNAc}_2\text{Man}_9$ and $\text{GlcNAc}_2\text{Man}_9\text{Glc}_1$. This result indicated that the fully assembled $\text{GlcNAc}_2\text{Man}_9\text{Glc}_3$ oligosaccharide was produced in the patient's cells, although in limited supply, and was successfully transferred onto nascent glycoproteins. $\text{GlcNAc}_2\text{Man}_9$ and $\text{GlcNAc}_2\text{Man}_9\text{Glc}_1$ arise from the trimming by glucosidases-I and -II, while $\text{GlcNAc}_2\text{Man}_9\text{Glc}_1$ can also arise from re-glucosylation during the process of protein folding (17). The $\text{GlcNAc}_2\text{Man}_8$ peak represents the oligosaccharide core trimmed by the ER α -mannosidase (18).

To support the hypothesis that the conversion of mannose-1-phosphate to GDP-mannose or to dolichylphosphate-mannose may be altered, the GDP-mannose pyrophosphorylase *GMPPA*, *GMPPB* genes and the dolichylphosphate-mannose synthase *DPM1*, *DPM2*, *DPM3* genes were screened by denaturing-HPLC (19). No mutation was found in any of these genes, thus ruling out a shortage of mannose substrates as a cause of the glycosylation disorder. This conclusion was supported by flow cytometry analysis where normal levels of the glycosylphosphatidylinositol (GPI)-anchored protein CD59, which also requires dolichylphosphate-mannose for the synthesis of the GPI-anchor, were found on the patient fibroblasts (data not shown).

To enable the detection of short LLOs that have escaped the established [^3H]mannose labeling procedure, we have adapted the recent method of Gao and Lehrman (20) to allow the efficient resolution of structures from *DolPP*- GlcNAc up to *DolPP*- $\text{GlcNAc}_2\text{Man}_9\text{Glc}_3$. Total LLOs were isolated from fibroblasts and hydrolyzed to liberate the glycan moiety. After a reductive amination with the fluorophore 2AB at their reducing termini, oligosaccharides were separated on a GlycoSep-N HPLC column, permitting the clear resolution of all oligosaccharide species found in LLOs. In the HPLC profile of patient FR, one can identify a peak comigrating with the external standard $\text{GlcNAc}(\beta 1,4)\text{GlcNAc}$ at 1.73 glucose units as compared with the elution positions of standard glucose oligomers (Fig. 3), whereas this peak is not detected in samples derived from control cells. The identity of this peak with $\text{GlcNAc}(\beta 1,4)\text{GlcNAc}$ was further confirmed by running

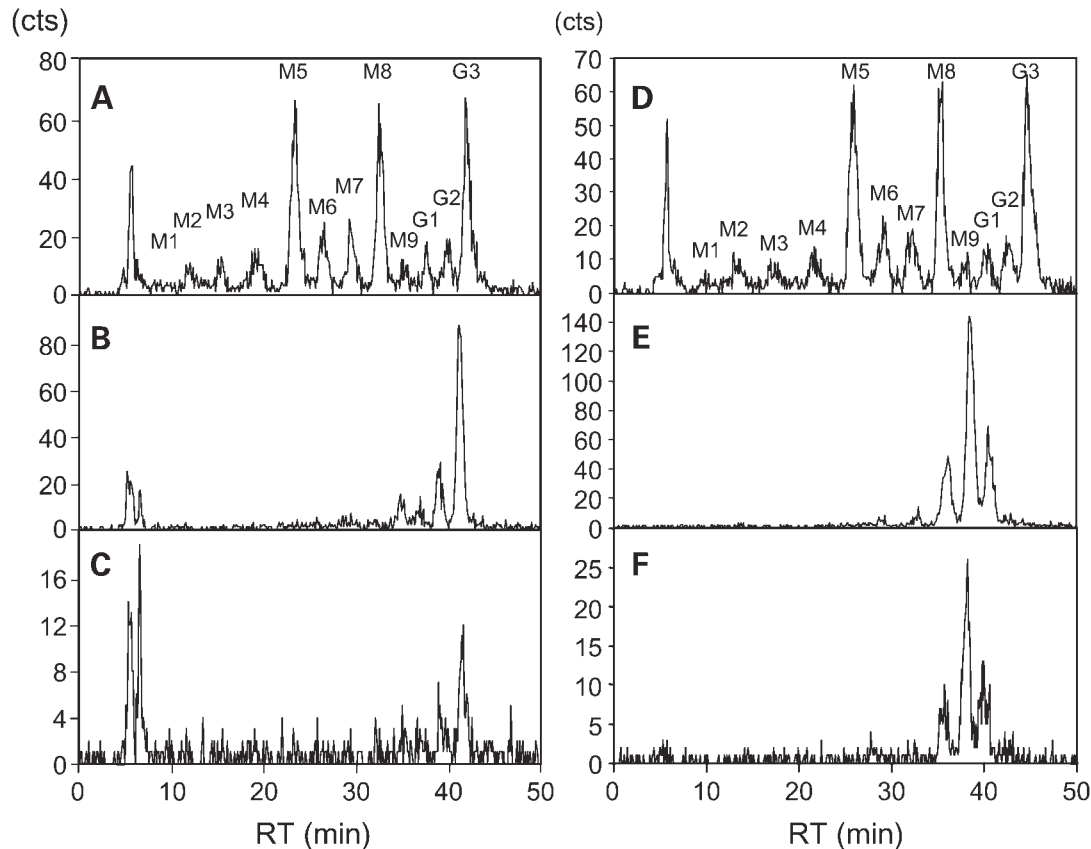


Figure 2. [^3H]Mannose-labeled lipid- and N-linked oligosaccharide profiles. HPLC separation of lipid-linked oligosaccharides from yeasts used as standard (**A**, **D**), of control human fibroblasts (**B**) and of patient FR fibroblasts (**C**) after metabolic labeling with [^3H]mannose for 60 min. Oligosaccharides were cleaved from dolichylpyrophosphate by mild acid hydrolysis and separated by HPLC. The identity of the oligosaccharide peaks ranging from GlcNAc₂Man₁ (M1) to GlcNAc₂Man₉Glc₃ (G3) is marked at the top of (**A**, **D**). [^3H]Mannose-labeled N-linked oligosaccharides from control cells (**E**) and from patient FR cells (**F**) were separated by HPLC after release from the protein by N-glycosidase F digestion. NLOs with peaks at M8, M9 and G1 are detected in control and patient fibroblasts.

collected fractions comprising the peak of interest onto a second dimension of reverse-phase HPLC (data not shown). This finding suggested an accumulation of *DolPP*-GlcNAc₂ to be at the origin of the glycosylation disorder in the patient FR. Also, in the GlycoSep-N HPLC profile of control fibroblasts, peaks corresponding to GlcNAc₂Man₉Glc₁₋₃ are visible. These peaks are also present in the patient HPLC profile, although with reduced intensity, thus confirming the limited supply of mature LLOs. The nature of the major peak, following the elution of chitobiose, is not known. However, after re-purification of the 2AB-labeled samples, the size of this peak relative to the chitobiose-peak was clearly reduced, indicative for non-carbohydrate, labeled material present in both fibroblast preparations (data not shown).

LLOs are assembled by the stepwise transfer of monosaccharides onto *DolPP*. The accumulation of *DolPP*-GlcNAc₂ implied a possible defect at the level of the addition of the first mannose residue catalyzed by the *ALG1* β 1,4 mannosyltransferase enzyme. The human *ALG1* gene has been characterized recently (21). It encodes a protein of 464 amino acids displaying 36% similarity to the yeast Alg1 enzyme (22). cDNAs encoding homologous proteins were found in all eukaryotic genomes searched, such as in

Caenorhabditis elegans and in *Drosophila melanogaster*. The sequence alignment of the putative *ALG1* proteins showed conserved domains, possibly regions representing the active site of the enzyme (Fig. 4). The *ALG1* cDNA was amplified by reverse transcription-PCR from control and from the patient FR fibroblasts. Sequencing of the DNA fragments revealed three heterozygous point mutations in the *ALG1* cDNA of patient FR (Fig. 4). These mutations, 450C>G, 773C>T and 1287T>A, lead to the amino acid substitutions S150R, S285L and D429E, respectively. These mutations were not found in control cDNAs sequenced in our laboratory, nor in 49 *ALG1* expressed sequence tags retrieved from GenBank (data not shown). The 450C>G, 773C>T and 1287T>A mutations detected in the patient FR are located on exons 4, 7 and 13 of the *ALG1* gene, respectively. Sequencing of these exons in DNA samples isolated from the parents of the patient FR indicated that the allele with S150R and D429E was inherited from the mother, whereas the S258L mutation stemmed from the father. The S258L mutation is located in a conserved domain of the *ALG1* protein. By contrast, the two maternal mutations do not correspond to positions conserved in the *ALG1* protein of the four species examined (Fig. 4).

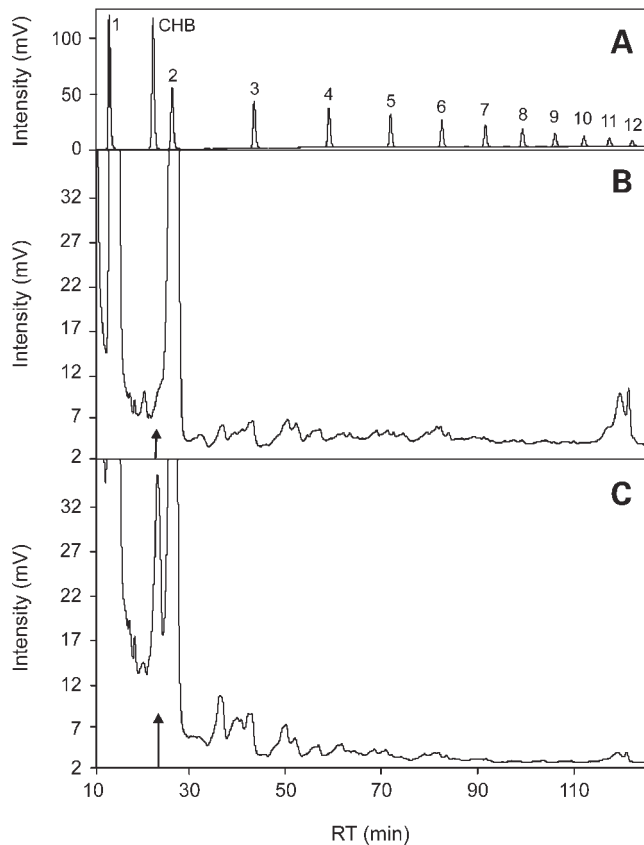


Figure 3. 2-Aminobenzamide-labeled oligosaccharide profiles on GlycoSep-N HPLC. (A) The separation of standard glucose oligomers with peaks corresponding to Glc₁ up to Glc₁₂, i.e. 1–12 glucose units. The external standard chitobiose (CHB), i.e. GlcNAc(β1,4)GlcNAc, elutes between Glc₁ and Glc₂ at 1.73 glucose units. The lipid-linked oligosaccharide profiles extracted from control (B) and patient FR (C) show in the FR sample a peak corresponding to the elution position of chitobiose, which is not visible in the control profile as indicated by the arrow.

To verify that the amino acid changes identified did impair *ALG1* protein function, the *ALG1* alleles of the patient FR and a normal human *ALG1* cDNA were introduced, under control of the strong glyceraldehyde phosphate dehydrogenase promoter (23), into *S. cerevisiae* yeasts carrying the *alg1-1* allele. This mutant allele does not support growth at elevated temperature due to reduced *ALG1* activity (24). As shown in Figure 5, growth at elevated temperature of the cell was restored by expressing the wild-type human *ALG1* cDNA. By contrast, the expression of the *ALG1* cDNAs isolated in the patient FR did not enable *alg1-1* yeasts to grow to the same extent as when transformed with the normal human *ALG1* cDNA. To examine the pathological impact of each of the two maternal mutations, we have transfected *alg1-1* yeasts with human *ALG1* cDNAs bearing the single mutation 450C>G [S150R] or the mutation 1287T>A [D429E]. The growth of *alg1-1* yeasts expressing the human *ALG1*[D429E] was similar to that of yeasts transfected with the wild-type human *ALG1* cDNA, indicating that this mutation likely represents a normal polymorphism. By comparison, the *ALG1* cDNA containing the 450C>G mutation failed to restore growth of *alg1-1* yeasts, which

supports the pathological impact of the maternal S150R substitution on *ALG1* function (Fig. 5). Taken together, this analysis confirmed the direct relationship between the *ALG1* mutations and the glycosylation disorder detected in the patient FR. Thus, the *ALG1* defect reported here represents a novel type of CDG, which should be referred to as CDG-Ik following the nomenclature rules established previously (11).

DISCUSSION

A new genetic disorder affecting the first mannose transfer of dolichol-linked oligosaccharide biosynthesis was identified in this study. The defects in this disorder are caused by mutations in the *ALG1* β1,4 mannosyltransferase gene. The *ALG1* protein catalyzes the transfer of the first mannose residue onto *DolPP*-GlcNAc₂ using GDP-mannose as donor substrate. This reaction takes place at the cytosolic side of the ER membrane, where dolichol-linked oligosaccharides are assembled to *DolPP*-GlcNAc₂Man₅. The latter is then translocated ('flipped') into the lumen of the ER, in a protein-assisted process which requires Rft1 protein (25). On the luminal side, the LLO assembly continues using dolichylphosphate-based donor substrates. The presence of the three terminal glucoses on LLOs is required for recognition by the oligosaccharyltransferase complex (26), although intermediate oligosaccharide structures can be transferred to nascent glycoproteins when the mature LLO is under limited supply. Usually under these conditions underglycosylation of proteins is observed as less sugar-chains are transferred. Such a transfer of incomplete oligosaccharides to proteins has been documented *in vivo* for example in yeast *alg* glycosylation mutants (27) as well as in CDG patients (28–30). Even though in the case of GlcNAc₂ transfer to protein has also been demonstrated *in vitro* (31) and *in vivo* in yeast (32), this occurs very inefficiently as it is a poor substrate for the oligosaccharyltransferase and, most likely, also for the flippase. Consequently, defects in GlcNAc₂Man₅ LLO assembly are severe and mostly lethal in yeast. The same severe phenotype can be expected in humans for complete blocks of LLO biosynthesis up to *DolPP*-GlcNAc₂Man₅. Even if transfer to protein would occur in patient cells, these truncated glycans are not expected to function properly in the glycan dependent quality control pathway (17,33). Also structures smaller than GlcNAc₂Man₃ would not support formation of complex type glycans. Therefore, the identification of glycosylation defects along the LLO cytosolic assembly line presupposes that the mutations encountered would only partially impair the activity of the corresponding glycosyltransferase enzymes. Yet, the resulting clinical manifestations can be quite severe, as observed in the patient reported in the present study.

Unlike the dolichylphosphate-mannose and -glucose dependent ER glycosyltransferases acting on lumenally oriented LLOs, which are highly hydrophobic proteins with multiple transmembrane domains, the *ALG1* sequence predicts a type-II transmembrane topology reminiscent of Golgi-localized glycosyltransferases. Alignment with homologous proteins in several eukaryotic species shows strong sequence conservation in the C-terminal half of *ALG1* proteins, suggesting the localization of the catalytic domain in this part of the protein. This is supported by significant homology with several prokaryotic

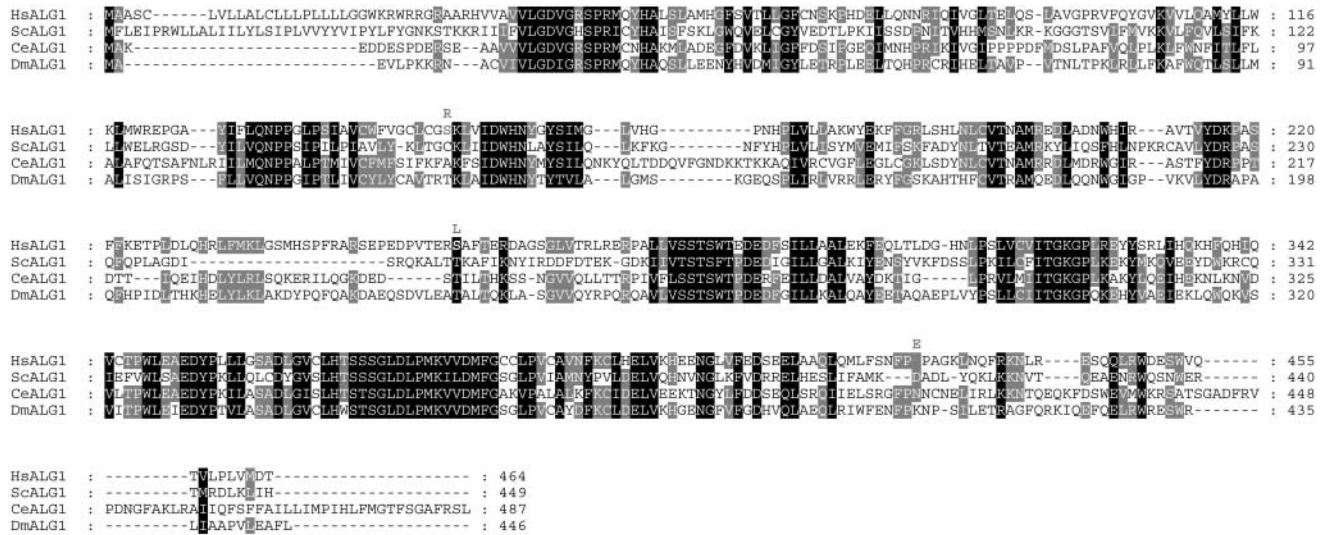


Figure 4. *ALG1* protein sequence comparison. Amino acid sequences of *ALG1* proteins from *Homo sapiens* (HsALG1), *S. cerevisiae* (ScALG1), *C. elegans* (CeALG1) and *D. melanogaster* (DmALG1) were aligned using the ClustalW program. Positions conserved in the four species are shaded in black, conserved in three species are shaded in gray. The amino acid changes S150R, S258L and D429E in patient FR are indicated above the human *ALG1* sequence. The electroferograms representing the corresponding mutations detected in the *ALG1* cDNA from patient FR are shown below the alignment. The maternal mutations S150R, D429E and the paternal S258L mutation are marked with arrows.

glycosyltransferase proteins in eubacteria (*Pseudomonas*, *Listeria*) and archae (*Methanosarcina*, *Methanococcus*). The three mutations identified in the patient FR were not at positions strictly conserved among *ALG1* protein homologs. This is consistent with a leaky phenotype as the observed residual activity in the patient fibroblasts enabled the formation of complete N-glycan chains on glycoproteins.

The *ALG1* deficiency represents the third type of CDG characterized by a defect of a cytosol-oriented ER-glycosyltransferase besides the *ALG2* mannosyltransferase (34) and *ALG7* N-acetylglucosamine-1-phosphotransferase (35) deficiencies, which have been assigned as CDG-Ii and -Ij, respectively. In these cases too, mutations lead to partial inactivation of the enzymes, resulting in low cellular pools of LLOs but to the transfer of complete oligosaccharides onto nascent glycoproteins.

For the *ALG1* patient studied here, the analysis of LLOs metabolically labeled with [3 H]mannose showed normal profiles, although with low incorporation yields, as observed in the cases of *ALG2* (34) and *ALG7* (35) deficiency described elsewhere. These findings are readily explained considering the enzymatic defects leading to the accumulation of LLO species containing no or at most two tritiated mannoses. Several CDG-I

patients remain untyped because the analysis of LLO profiles obtained from their fibroblasts only shows low [3 H]mannose incorporation and a peak corresponding to the complete *DolPP*-GlcNAc₂Man₉Glc₃ structure. Thus, the efficient detection of accumulating short LLO structures requires the establishment of alternative methods. Gao and Lehrman (20) have recently presented a procedure allowing the quantitative detection of total LLOs from cell cultures and animal tissues without metabolic labeling. However, to permit separation and detection of all LLO structures in a single run, we separated 2AB-derivatized oligosaccharides using a GlycoSep-N HPLC column. As shown in the present study, this method allows the quantitative detection of short LLO species without the use of radioactive isotopes, which was critical for the diagnosis of the *ALG1* mannosyltransferase deficiency. The availability of this alternative LLO analysis procedure should enable the identification of further CDG-I types uncharacterizable using the radioactive method. Besides the medical importance of establishing a precise diagnostic, the characterization of CDG-I cases associated with the accumulation of short LLOs may help identification of still undiscovered glycosyltransferase genes along the ER-glycosylation pathway. This includes the UDP-GlcNAc:*DolPP*-GlcNAc β 1,4 N-acetylglucosaminyltransferase

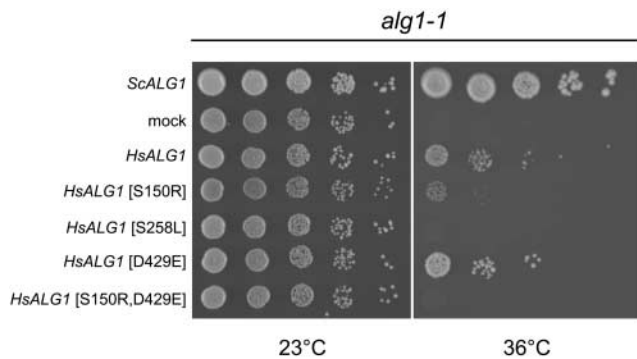


Figure 5. Complementation of *alg1-1* yeasts with human *ALG1* cDNAs. Transformants were spotted in 10-fold dilutions on YPD plates and incubated at 23 or 36°C for 90 h. Transformation was performed with the vector alone (mock), the pMJ-*ALG1* vector expressing the *S. cerevisiae* *ALG1* gene (*ScALG1*) and with plasmids expressing normal human *ALG1* cDNA (*HsALG1*), the mutated cDNAs from patient FR (*HsALG1*[S150R, D429E], *HsALG1*[S258L]) and *ALG1* cDNAs containing the isolated S150R and D429E mutations.

and the GDP-mannose:*Dol*PP-GlcNAc₂Man₂ α1,6 mannosyl-transferase genes.

MATERIALS AND METHODS

Glycan extraction

Patient primary fibroblasts were isolated from a skin biopsy and grown in Dulbecco's modified Eagle's medium (DMEM/F12, Gibco) with 4.5 g/l glucose and 10% fetal calf serum. Fibroblasts (5×10^7) were cultured to 90% of confluence and metabolically labeled with 150 μCi [³H]mannose (54.0 Ci/mmol, Amersham Bioscience) as described previously (30). Short LLOs were extracted twice from the cell pellets with 3 ml of chloroform-methanol 3:2 (v/v). Long LLOs were then further extracted twice with 3 ml of chloroform-methanol-water 10:10:3 (v/v/v). N-linked oligosaccharides (NLOs) were recovered from the solid cell pellet after LLO extraction by N-glycosidase F digestion (30).

HPLC analysis of [³H]mannose-labeled oligosaccharides

[³H]Mannose-labeled oligosaccharides were separated on a 250 × 4.6 mm LC-NH₂ aminopropyl column (Supelco), equipped with an LC-NH₂ guard column, as described previously (36).

Hydrolysis and cleanup of total cellular LLOs

Total cellular LLOs were extracted from 5×10^7 fibroblasts as described above but without metabolic labeling. Dried LLOs were hydrolyzed with 2 ml of 0.1 M HCl in 50% isopropyl alcohol at 50°C for 60 min to release pyrophosphate-linked oligosaccharides (20). Hydrolysates were neutralized by adding 1 ml of 0.2 M NaOH and residual salt and lipids were removed by loading the samples onto C₁₈ SepPak columns (Waters) directly connected to 3 ml ENVI-CARB solid phase extraction tubes (Supelco). These combined columns were conditioned with 5 ml of methanol, followed by 5 ml of acetonitrile and 5 ml

H₂O-acetonitrile (3:1, v/v) then equilibrated with 9 ml of 2% acetonitrile/0.1 M ammonium acetate in H₂O. After loading of the samples, the columns were washed with 9 ml of the same solution. For elution of the oligosaccharides, the C₁₈ SepPak cartridge was removed and glycans were eluted from the ENVI-CARB tube with 6 ml of H₂O-acetonitrile (3:1, v/v).

2-Aminobenzamide labeling of oligosaccharides and HPLC separation

The dried samples were labeled with 2AB according to Bigge *et al.* (37). The glycan cleanup was performed using the paper disk method as described in Merry *et al.* (38). The separation of 2AB-labeled N-glycans was performed by HPLC using a GlycoSep-N column (Glyko Inc.) according to Royle *et al.* (39) but modified to a three solvent system suitable for low pressure gradient mixing. Solvent A was 10 mM formic acid adjusted to pH 4.4 with ammonia solution in 80% acetonitrile. Solvent B was 30 mM formic acid adjusted to pH 4.4 with ammonia solution in 40% acetonitrile. Solvent C was 0.5% formic acid. The column temperature was 30°C. Gradient conditions were a linear gradient of 100% A to 100% B over 160 min at a flow rate of 0.4 ml/min, followed by 2 min 100% B to 100% C, increasing the flow rate to 1 ml/min. The column was washed for 5 min with 100% C, returning to 100% A over 2 min and running for 6 min at 100% A at a flow rate of 1 ml/min, then returning the flow rate to 0.4 ml/min for 5 min. Samples were injected in water.

Reverse transcription-PCR

RNA was extracted from 2×10^7 fibroblasts using Tri-reagent (Sigma) following the instructions of the manufacturer. cDNA was synthesized from 5 μg of total RNA using the specific *ALG1* reverse primer 5'-AACGCAGGATGAGGAGTTCT-3', with 5% DMSO and 1 unit of reverse transcriptase ImPromII (Promega). The synthesis was performed at 42°C for 120 min. The protein coding region was amplified by PCR using 3 μl of RT product and the primers 5'-ATGGCGGCCTCATGCTTGGT-3' and 5'-CCAAGCCTCCAGAGAAGA-3'. The cycling conditions were 30 cycles at 94°C for 45 s, 55°C for 30 s and 72°C for 2 min. Primers and unincorporated nucleotides were eliminated using Qiaquick columns (Qiagen) and the PCR products were directly sequenced (Microsynth, Balgach, Switzerland).

Genomic DNA PCR

Genomic DNA from patient FR and from his parents was isolated from 10^7 fibroblasts and from 5 ml of blood, respectively. *ALG1* exons 4, 7 and 13 were amplified with the primers 5'-CCATCACCAGCTGTTGTTAG-3', 5'-CAGCATTC-GGCCTGATTAC-3', 5'-TGGCCTGGTGCTGCTGATGT-3', 5'-AGCTGCCAGCAGGATGGAGA-3' and 5'-GTGGCTGC-TTGCCAGCTTA-3', 5'-CGAAGAGCACTTGGAGATG-3', respectively, for 30 cycles at 94°C for 45 s, 54°C for 30 s and 72°C for 1.5 min. The PCR products were directly sequenced (Microsynth, Balgach, Switzerland) after removal of the primers and unincorporated nucleotides. The other genes were screened essentially as described before (19).

Plasmid construction

The human *ALG1* cDNAs from healthy control, the S150R/D429E allele and the S258L allele from patient fibroblasts were amplified with the primers 5'-ACTGCTGCGGAGGATCCAA-GATGGC-3' and 5'-CCAAGCCTCCAGAGAAGA-3', which contain a *Bam*HI or a *Hind*III restriction site (underlined). The fragments were subcloned into the *Bam*HI and *Hind*III sites of pBluescript-II SK⁺ (Stratagene). From there, the human *ALG1* cDNAs from patient FR and from control fibroblasts were subcloned as *Bam*HI-*Xho*I fragments into p426GDP (40). To separate the S150R and D429E mutations, 467 bp *Eco*RI-*Pae*I or 58 bp *Kpn*2I-*Bpu*1102I fragments of the patient's S150R/D429E cDNA were replaced by the corresponding fragments from normal human *ALG1* cDNA. The resulting plasmids are multicopy yeast vectors with the *ALG1* cDNAs under control of the *TDH3* promoter. The plasmid pMJ-*ALG1*, a single-copy plasmid with the yeast *ALG1* gene under control of its own promoter, was created from pRS316-NES17 (41) by digestion with *Eco*RI followed by self-ligation.

Yeast strains and media

S. cerevisiae strains used in this study were derivatives of K57-6C (*MATa alg1-1 ura3-52 mal gal2 can(r)*) (ATCC 208304). Standard yeast media and genetic techniques were used (42). Yeast cells were transformed using the LiOAc/PEG method and the transformants were selected on solid medium lacking uracil. Strain K57-6C and its transformants were propagated at 23°C.

Complementation of ALG1 mutants

Strain K57-6C and its transformants were grown in liquid medium lacking uracil but supplemented with 1 M sorbitol. For the spot assay, 5 µl of serial 10-fold dilutions of K57-6C transformants were spotted on full medium (YPD), starting at 5×10^5 cells. Plates were incubated at a given temperature for 90 h.

ACKNOWLEDGEMENTS

The authors would like to thank Mathias Jenal (ETH Zürich) for technical assistance and Jonne Helenius for comments on the manuscript. This work was supported by the TANDEM grant 3238-069471.02 and the SNF professorship grant 631-062662.00 from the Swiss National Foundation and by the European Union (QLG1-CT-2000-00047).

REFERENCES

- Keir, G., Winchester, B.G. and Clayton, P. (1999) Carbohydrate-deficient glycoprotein syndromes: inborn errors of protein glycosylation. *Ann. Clin. Biochem.*, **36**, 20–36.
- Krasnewich, D. and Gahl, W.A. (1997) Carbohydrate-deficient glycoprotein syndrome. *Adv. Pediatr.*, **44**, 109–140.
- Jaeken, J. and Matthijs, G. (2001) Congenital disorders of glycosylation. *A. Rev. Genomics Hum. Genet.*, **2**, 129–151.
- Beltran-Valero de Bernabe, D., Currier, S., Steinbrecher, A., Celli, J., van Beusekom, E., van der Zwaag, B., Kayserili, H., Merlini, L., Chitayat, D., Dobyns, W.B. *et al.* (2002) Mutations in the *O*-mannosyltransferase gene POMT1 give rise to the severe neuronal migration disorder Walker-Warburg syndrome. *Am. J. Hum. Genet.*, **71**, 1033–1043.
- Yoshida, A., Kobayashi, K., Many, H., Taniguchi, K., Kano, H., Mizuno, M., Inazu, T., Mitsuhashi, H., Takahashi, S., Takeuchi, M. *et al.* (2001) Muscular dystrophy and neuronal migration disorder caused by mutations in a glycosyltransferase, POMGnT1. *Dev. Cell*, **1**, 717–724.
- Kobayashi, K., Nakahori, Y., Miyake, M., Matsumura, K., Kondo-Iida, E., Nomura, Y., Segawa, M., Yoshioka, M., Saito, K., Osawa, M. *et al.* (1998) An ancient retrotransposal insertion causes Fukuyama-type congenital muscular dystrophy. *Nature*, **394**, 388–392.
- Longman, C., Brockington, M., Torelli, S., Jimenez-Mallebrera, C., Kennedy, C., Khalil, N., Feng, L., Saran, R.K., Voit, T., Merlini, L. *et al.* (2003) Mutations in the human LARGE gene cause MDC1D, a novel form of congenital muscular dystrophy with severe mental retardation and abnormal glycosylation of α -dystroglycan. *Hum. Mol. Genet.*, **12**, 2853–2861.
- Quentin, E., Gladen, A., Roden, L. and Kresse, H. (1990) A genetic defect in the biosynthesis of dermatan sulfate proteoglycan: galactosyltransferase I deficiency in fibroblasts from a patient with a progeroid syndrome. *Proc. Natl Acad. Sci. USA*, **87**, 1342–1346.
- Kitagawa, H., Shimakawa, H. and Sugahara, K. (1999) The tumor suppressor EXT-like gene EXT2 encodes an α 1,4-*N*-acetylhexosaminyltransferase that transfers *N*-acetylgalactosamine and *N*-acetylglucosamine to the common glycosaminoglycan–protein linkage region. The key enzyme for the chain initiation of heparan sulfate. *J. Biol. Chem.*, **274**, 13933–13937.
- de Jong, G. and van Eijk, H.G. (1988) Microheterogeneity of human serum transferrin: a biological phenomenon studied by isoelectric focusing in immobilized pH gradients. *Electrophoresis*, **9**, 589–598.
- Aebi, M., Helenius, A., Schenk, B., Barone, R., Fiumara, A., Berger, E.G., Hennet, T., Imbach, T., Stutz, A., Bjursell, C. *et al.* (1999) Carbohydrate-deficient glycoprotein syndromes become congenital disorders of glycosylation: an updated nomenclature for CDG. First International Workshop on CDGS. *Glycoconj. J.*, **16**, 669–671.
- Aebi, M. and Hennet, T. (2001) Congenital disorders of glycosylation: genetic model systems lead the way. *Trends Cell Biol.*, **11**, 136–141.
- Freeze, H.H. (2001) Update and perspectives on congenital disorders of glycosylation. *Glycobiology*, **11**, 129R–143R.
- Panneerselvam, K. and Freeze, H.H. (1996) Mannose corrects altered N-glycosylation in carbohydrate-deficient glycoprotein syndrome fibroblasts. *J. Clin. Invest.*, **97**, 1478–1487.
- Burda, P., Borsig, L., de Rijk-van Andel, J.F., Wevers, R.A., Jaeken, J., Carchon, H., Berger, E.G. and Aebi, M. (1998) A novel carbohydrate-deficient glycoprotein syndrome characterized by a deficiency in glucosylation of the dolichol-linked oligosaccharide. *J. Clin. Invest.*, **102**, 647–652.
- Imbach, T., Schenk, B., Schollen, E., Burda, P., Stutz, A., Grünewald, S., Bailie, N., King, M.D., Jaeken, J., Matthijs, G. *et al.* (2000) Deficiency of dolichol-phosphate-mannose synthase-1 causes congenital disorder of glycosylation (CDG) type-Ie. *J. Clin. Invest.*, **105**, 233–239.
- Parodi, A.J. (1999) Reglucosylation of glycoproteins and quality control of glycoprotein folding in the endoplasmic reticulum of yeast cells. *Biochim. Biophys. Acta*, **1426**, 287–295.
- Camirand, A., Heysen, A., Grondin, B. and Herscovics, A. (1991) Glycoprotein biosynthesis in *Saccharomyces cerevisiae*. Isolation and characterization of the gene encoding a specific processing α -mannosidase. *J. Biol. Chem.*, **266**, 15120–15127.
- Schollen, E., Martens, K., Geuzens, E. and Matthijs, G. (2002) DHPLC analysis as a platform for molecular diagnosis of congenital disorders of glycosylation (CDG). *Eur. J. Hum. Genet.*, **10**, 643–648.
- Gao, N. and Lehrman, M.A. (2002) Analyses of dolichol pyrophosphate-linked oligosaccharides in cell cultures and tissues by fluorophore-assisted carbohydrate electrophoresis. *Glycobiology*, **12**, 353–360.
- Takahashi, T., Honda, R. and Nishikawa, Y. (2000) Cloning of the human cDNA which can complement the defect of the yeast mannosyltransferase I-deficient mutant *alg 1*. *Glycobiology*, **10**, 321–327.
- Albright, C.F. and Robbins, R.W. (1990) The sequence and transcript heterogeneity of the yeast gene *ALG1*, an essential mannosyltransferase involved in N-glycosylation. *J. Biol. Chem.*, **265**, 7042–7049.
- Bitter, G.A., Chang, K.K. and Egan, K.M. (1991) A multi-component upstream activation sequence of the *Saccharomyces cerevisiae* glyceraldehyde-3-phosphate dehydrogenase gene promoter. *Mol. Gen. Genet.*, **231**, 22–32.
- Huffaker, T.C. and Robbins, P.W. (1982) Temperature-sensitive yeast mutants deficient in asparagine-linked glycosylation. *J. Biol. Chem.*, **257**, 3203–3210.

25. Helenius, J., Ng, D.T., Marolda, C.L., Walter, P., Valvano, M.A. and Aebi, M. (2002) Translocation of lipid-linked oligosaccharides across the ER membrane requires Rft1 protein. *Nature*, **415**, 447–450.
26. Burda, P. and Aebi, M. (1999) The dolichol pathway of N-linked glycosylation. *Biochim. Biophys. Acta*, **1426**, 239–257.
27. Huffaker, T.C. and Robbins, P.W. (1983) Yeast mutants deficient in protein glycosylation. *Proc. Natl Acad. Sci. USA*, **80**, 7466–7470.
28. Körner, C., Knauer, R., Stephani, U., Marquardt, T., Lehle, L. and von Figura, K. (1999) Carbohydrate deficient glycoprotein syndrome type IV: deficiency of dolichyl-P-Man:Man(5)GlcNAc(2)-PP-dolichyl mannosyltransferase. *EMBO J.*, **18**, 6818–6822.
29. Schenk, B., Imbach, T., Frank, C.G., Grubenmann, C.E., Raymond, G.V., Hurvitz, H., Raas-Rotschild, A., Luder, A.S., Jaeken, J., Berger, E.G. et al. (2001) *MPDUL* mutations underlie a novel human congenital disorder of glycosylation (CDG), designated type If. *J. Clin. Invest.*, **108**, 1687–1695.
30. Grubenmann, C.E., Frank, C.G., Kjaergaard, S., Berger, E.G., Aebi, M. and Hennot, T. (2002) *ALG12* mannosyltransferase defect in congenital disorder of glycosylation type-Ig. *Hum. Mol. Genet.*, **11**, 2331–2339.
31. Sharma, C.B., Lehle, L. and Tanner, W. (1981) N-Glycosylation of yeast proteins. Characterization of the solubilized oligosaccharyl transferase. *Eur. J. Biochem.*, **116**, 101–108.
32. Cueva, R., Cotano, C. and Larriba, G. (1998) N-glycosylation by transfer of GlcNAc2 from dolichol-PP-GlcNAc2 to the protein moiety of the major yeast exoglucanase. *Yeast*, **14**, 773–781.
33. Helenius, A. and Aebi, M. (2001) Intracellular functions of N-linked glycans. *Science*, **291**, 2364–2369.
34. Thiel, C., Schwarz, M., Peng, J., Grzmil, M., Hasilik, M., Bräulke, T., Kohlschütter, A., von Figura, K., Lehle, L. and Körner, C. (2003) A new type of congenital disorders of glycosylation (CDG-II) provides new insights into the early steps of dolichol-linked oligosaccharide biosynthesis. *J. Biol. Chem.*, **278**, 22498–22505.
35. Wu, X., Rush, J.S., Karaoglu, D., Krasnewich, D., Lubinsky, M.S., Waechter, C.J., Gilmore, R. and Freeze, H.H. (2003) Deficiency of UDP-GlcNAc:dolichol Phosphate N-acetylglucosamine-1 phosphate transferase (DPAGT1) Causes a novel congenital disorder of glycosylation Type Ij. *Hum. Mutat.*, **22**, 144–150.
36. Zufferey, R., Knauer, R., Burda, P., Stagljar, I., te Heesen, S., Lehle, L. and Aebi, M. (1995) STT3, a highly conserved protein required for yeast oligosaccharyl transferase activity *in vivo*. *EMBO J.*, **14**, 4949–4960.
37. Bigge, J.C., Patel, T.P., Bruce, J.A., Goulding, P.N., Charles, S.M. and Parekh, R.B. (1995) Nonselective and efficient fluorescent labeling of glycans using 2-amino benzamide and anthranilic acid. *Anal. Biochem.*, **230**, 229–238.
38. Merry, A.H., Neville, D.C., Royle, L., Matthews, B., Harvey, D.J., Dwek, R.A. and Rudd, P.M. (2002) Recovery of intact 2-aminobenzamide-labeled O-glycans released from glycoproteins by hydrazinolysis. *Anal. Biochem.*, **304**, 91–99.
39. Royle, L., Mattu, T.S., Hart, E., Langridge, J.I., Merry, A.H., Murphy, N., Harvey, D.J., Dwek, R.A. and Rudd, P.M. (2002) An analytical and structural database provides a strategy for sequencing O-glycans from microgram quantities of glycoproteins. *Anal. Biochem.*, **304**, 70–90.
40. Mumberg, D., Müller, R. and Funk, M. (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene*, **156**, 119–122.
41. Shimma, Y., Nishikawa, A., bin Kassim, B., Eto, A. and Jigami, Y. (1997) A defect in GTP synthesis affects mannose outer chain elongation in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.*, **256**, 469–480.
42. Berlin, V., Brill, J.A., Trueheart, J., Boeke, J.D. and Fink, G.R. (1991) Genetic screens and selections for cell and nuclear fusion mutants. *Meth. Enzymol.*, **194**, 774–792.